Optimising the settlement and hatchery culture of Saccharina latissima (Phaeophyta) by manipulation of growth medium and substrate surface condition

Kerrison, Philip D.; Stanley, Michele S.; Kelly, Maeve; Macleod, Adrian; Black, Kenneth D.; Hughes, Adam D.

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Corresponding Author:	Philip D Kerrison Scottish Association for Marine Science Dunbeg, Argyll UNITED KINGDOM
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Scottish Association for Marine Science
Corresponding Author's Secondary Institution:	
First Author:	Philip D Kerrison
First Author Secondary Information:	
Order of Authors:	Philip D Kerrison
	Michele S Stanley
	Maeve Kelly
	Adrian MacLeod
	Kenneth D Black
	Hughes D Adam
Order of Authors Secondary Information:	
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Optimising the settlement and hatchery culture of *Saccharina latissima* (Phaeophyta) by manipulation of growth media and substrate surface condition

Philip D Kerrison[1,2]

Michele S Stanley[1]

Maeve Kelly[1]

Adrian MacLeod[3]

Kenneth D Black[1]

Adam D Hughes[1]

[1] SAMS, Scottish Marine Institute, Oban, Argyll, UK, PA37 1QA.

[2] Corresponding author: Email: Philip.Kerrison@sams.ac.uk, Telephone:+44 (0)1631 559309, Fax: :+44 (0)1631 559309.

[3]SAMS Research Sciences Ltd (SRSL), Scottish Marine Institute, Oban, Argyll, UK, PA37 1QA.

Abstract

The Phaeophyte macroalgae *Saccharina latissima* is gaining economic importance as an aquaculture crop. To decrease costs associated with the hatchery, the time required for meiospores to develop into sporophytes ready for outplanting must be minimised and survivorship maximised. The settlement and juvenile development of *S. latissima* was examined in a series of experimental manipulations. It was determined that: 1) Meiospore settlement should be conducted in the dark in nutrient enriched media. 2) Continued nutrient enrichment in the hatchery increased growth and survival of the developing sporophytes. 3) It is best to use the diatom inhibitor germanium dioxide

(GeO_2) only during settlement and the first week of light exposure, rather than continuously or not at all. This treatment leads to the highest survival rate and sporophyte length. 4) Pre-treating the settlement surface with a commercial yeast extract can increase settlement and early development size, however over-application can be highly detrimental leading to reduced survival, size and patchy growth.

KEYWORDS: *Saccharina latissima*; germanium dioxide; hatchery; settlement; cultivation; nutrient

Introduction

Saccharina latissima is a fast-growing Phaeophyte macroalgae which has economic value as an aquaculture crop. It may be grown as a monoculture, or as a component in an integrated multi-trophic aquaculture system where it can benefit from the additional dissolved nutrients released into the water by co-cultured animals such as *Salmo salmar* and *Mytilus edulis* (Wang et al. 2014; Sanderson et al. 2008; Rößner et al. 2014). The lifecycle of *S. latissima* is a heteromorphic alteration of generations: The diploid sporophytes release meiospores which settle and develop into microscopic single sex haploid gametophytes. Following fertilisation, an embryonic sporophyte develops, which continues to grow to adult size over the following months or year (Schiel and Foster 2006).

This lifecycle is manipulated for aquaculture, using the method developed in China for *Saccharina japonica* (FAO 2004). Meiospores are extracted from fertile sporangial tissue, and then allowed to settle onto string or twine on spools within enclosed tanks. During early development *in situ*, the microscopic stages are at risk of being eliminated by grazers or overgrown and outcompeted by fouling organisms. To improve survivorship, the seeded twine is cultured under controlled conditions within a hatchery until sporophytes are 2-10 mm. These are then deployed to a coastal aquaculture site. Ideally, the hatchery stage should maximise meiospore settlement, minimise the sporophyte

developmental time and result in a dense coverage of healthy young sporophytes which have high resilience to the fluctuating environmental parameters following outplanting. This may be achievable through simple manipulation of the hatchery conditions such as light, nutrients, removal of competitors, twine pre-treatment or surface roughness manipulation.

The juvenile stages of *S. latissima* and other kelps are often cultured at irradiances between 20-100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (tom Dieck 1993; Hanelt et al. 1997; Shea and Chopin 2007), but few have investigated the effect of light on meiospore settlement. High light or long exposure times can lead to reduced settlement or germination in both *Macrocystis pyrifera* and *Pterygophora californica* (Graham 1996; Cie and Edwards 2008). Meiospore germination is known to occur in both light and darkness (Han et al. 2011; Huovinen et al. 2000) and darkness has been used previously for meiospore settlement in *S. latissima* (Shea and Chopin 2007; Flavin et al. 2013). Nevertheless, the authors are not aware of any reports showing whether darkness is preferable during settlement.

Inorganic nutrients are essential for the growth of phototrophic organisms. Nutrient concentration also influences the behaviour and settlement of meiospores, the development of gametophytes and the growth rate of sporophytes (Reed et al. 1999; Amsler and Neushul 1990; Kinlan et al. 2003; Morelisen et al. 2013; Chapman et al. 1978), with higher concentrations generally favoured. To optimise sporophyte development, sufficient nutrients must be supplied to them within the kelp hatchery, without concentrations becoming inhibitory (Amsler and Neushul 1989) or encouraging the growth of fouling organisms. Nutrient addition may be achieved through either constant or intermittent refreshment of the tanks with nutrient rich seawater or the addition of prepared nutrients (Forbord et al. 2012).

There is a significant threat that the microscopic stages of kelp will be overgrown by the proliferation of benthic diatoms while in the hatchery, leading to patchy survival or their complete eradication (P.

77 Kerrison unpublished results). To prevent this, germanium dioxide (GeO_2) treatment has been
78 recommended at concentrations between 0.01-0.50 mL of saturated GeO_2 solution L^{-1} of seawater
79 (Markham and Hagmeier 1982; Shea and Chopin 2007). GeO_2 interferes with the formation of the
80 diatom's silica frustule inhibiting their growth (Lewin 1966), but also has an inhibitory effect on the
81 growth of Phaeophyte macroalgae (Markham and Hagmeier 1982). The mechanism for this toxicity
82 is currently unknown but two hypotheses have been suggested. Firstly, silica deposition has been
83 documented in *S. japonica* and may have a protective function (Mizuta and Yasui 2012). Therefore,
84 Phaeophyte inhibition may have the same physiological basis as in diatoms. However, silica is not
85 normally considered an essential element for the Phaeophyceae and can be omitted from culture
86 media with no apparent deleterious effects (McLachlan et al. 1971). Secondly, Tarahovskaya *et al*.
87 (2012) showed that GeO_2 interferes with growth and development in the Phaeophyte *Fucus*
88 *vesiculosus* leading to morphological abnormalities. The authors suggest that this action maybe due
89 to Ge substituting for Boron within various complexes, which is known to have deleterious effects in
90 land plants (McIlrath and Skok 1966). Regardless of the mechanism, the beneficial inhibition of
91 diatoms by GeO_2 is balanced against its potential to negatively impact macroalgal development.
92 Determining the ideal GeO_2 application method is important to optimising hatchery growth.
93
94 When a surface is immersed in seawater it immediately begins to absorb macromolecules, such as
95 proteins and polysaccharide, which adhere reversibility to the surface (Lejars et al. 2012). This is
96 known as surface conditioning and creates a complex chemical topography altering the surface
97 characteristics, and in turn, the settlement of organisms (Thome et al. 2012). Pre-treatment of the
98 settlement surface with certain organic compounds, i.e. polylysine and D-glucose, has been shown
99 to significantly increase settlement in a number of algal species (Lee et al. 2008; Santelices and Aedo
100 1999). Such compounds are of commercial interest, by facilitating settlement within the hatcheries
101 of aquaculture species. The commercially available yeast extract Marmite© is a complex mixture of
102 11.8 % carbohydrate, 23.4 % ash and 28 % protein including 1.9% lysine (www.Foodcomp.dk). This

chemical complexity, combined with its low price and high availability as a commercial condiment may make it an effective pre-treatment to increase hatchery settlement of meiospores by conditioning the settlement surface.

The effect of substrate roughness on the settlement of the spores of fouling macroalgae, usually *Ulva* spp., has been extensively studied with the aim to develop more effective antifouling coatings. Surface irregularities increase the surface area and so the number of attachment sites (Fletcher and Callow 1992). This can lead to a tenfold increase in settlement per unit area within specific microenvironments (Callow et al. 2002; Fletcher and Callow 1992). Conversely, specific microtopographical features can reduce or deter fouling, with a 86 % decrease in *Ulva* settlement observed on a Shark scale biomimetic topography (Carman et al. 2006). It is unclear whether surface roughness affects the settlement and attachment of kelp meiospores. Exposure to a high velocity flow or spray, has often been used before to determine attachment tenacity (Finlay et al. 2002; Cassé et al. 2007).

The aim of this study is to determine whether the settlement and early development of *S. latissima* can be optimised through simple manipulation of the hatchery environment. Through a series of experiments we investigate:

1. How settlement is influenced by light or darkness, nutrient enrichment the presence of germanium dioxide, yeast extract and surface roughness.
2. How these factors influence the survival and development rate of *S. latissima* juvenile stages.
3. From these results, we determine the hatchery conditions which result in the shortest development time and the largest final size.

128 **Materials and Methods**

129 Three experiments were designed to test the effect of five variables on *S. latissima* development
130 during the hatchery phase: light/dark, nutrients, germanium dioxide (GeO_2), a pre-treatment with a
131 yeast extract and roughness (Fig. 1). In addition, a flume was utilised to examine whether
132 attachment success was influenced.

133

134 For each experiment, five fertile specimens of *S. latissima* were collected at low tide. The sporangial
135 areas were cut from the thalli, rinsed with Tyndallised seawater (Kawachi and Noël 2005) and then
136 wiped firmly until dry, using laboratory tissues (Kimtech, UK), to remove epiphytes. This was
137 repeated 4-5 times. The material was then cut into 1-2 cm^2 pieces and left overnight in a refrigerator
138 at 4 °C. The following morning, these were placed in 8.5 °C Tyndallised seawater (salinity 33 psu)
139 enriched with F/2 without silicate (herein referred to as F/2) and incubated in the dark for one hour
140 to induce meiospore release. The media was agitated every 15 minutes to encourage the process
141 (Gordon and Brawley 2004). The resultant solution was passed through a 50 µm filter and kept in
142 motion using a magnetic mixer while the meiospore concentration was determined using a Sedgwick
143 Rafter counting chamber.

144

145 **Experiment one (E1)**

146 To test the effects of light on settlement, 100,000 meiospores were distributed into Petri dishes
147 containing 20 mL F/2 and a glass microscope slide (76x25 mm). The slides were pre-cleaned with: a
148 24 hour soak in 5 % Decon90, followed by 24 hours in 10 % hydrochloric acid, then rinsed thoroughly
149 in ultra-high purity water and finally dried at 40 °C. These dishes were incubated at 8.5 °C for 48
150 hours without agitation to allow settlement under two conditions: darkness or illuminated by blue
151 fluorescent lighting at 15-25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ 12:12 L:D (n=5; Fig 1). At the end of this period, each slide
152 was placed into a fresh Petri dish with 20 mL of F/2 to rinse away unattached meiospores.

Epifluorescent microscopy was used to determine the meiospore settlement density (cells·mm^{-2}). This employed an Axioskop 2 microscope, a UV light source and filter set 09 (Zeiss, Germany).

Experiment two (E2)

100,000 meiospores were distributed into Petri dishes containing 20 mL of F/2 and a pre-cleaned glass slide. These were allowed to settle for 48 hours in the dark at 8.5 °C without agitation. Seven variations were made to test the effects of: nutrients, GeO_2, pre-treatment with a yeast extract and surface roughness (n=5; see Fig 1):

1. With nutrients (F/2). Settlement in F/2.
2. No nutrients (NW): Settlement in seawater only.
3. With nutrients and diatom inhibitor (F/2+GeO_2): Settlement in F/2 media containing 0.56 mg·L^{-1} of germanium dioxide (0.125 mL of a saturated solution·L^{-1}).
4. With nutrients and surface pre-treatment (F/2+PT): The slides were thinly coated in the yeast extract (1.6±0.5 mg·cm^{-2}; Marmite©, Unilever plc, UK). This was allowed to dry for 2 hours in a laminar flow cabinet before settlement in F/2.
5. With nutrients and light roughness (F/2 + L rgh): Slide roughened using sandpaper with a mean particle size of 100 µm. Settlement in F/2.
6. With nutrients and medium roughness (F/2 + M rgh). Roughened using sandpaper with a mean particle size of 200 µm. Settlement in F/2.
7. With nutrients and coarse roughness (F/2 + C rgh). Roughened using sandpaper with a mean particle size of 400 µm. Settlement in F/2.

Following the 48 hours settlement phase, slides were transferred to new Petri dishes of fresh media to rinse away unattached meiospores. These were kept in low light at <12 °C for up to two hours. One set of samples were directly examined by fluorescent microscopy to the determine settlement

density. The second set were secured in the test section of a 3.5 m fibreglass flume (Macleod 2013), and exposed to a turbulent flow velocity of 1 $m \cdot s^{-2}$ for five minutes. Following this, the slides were removed and examined using fluorescent microscopy.

A separate set of samples were transferred into borosilicate basins containing 150 mL of the respective media with gentle bubbling of 0.45 µm filtered air (Whatman, UK). These were illuminated by blue fluorescent lighting at 15-25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ 12:12 L:D for a further three weeks with weekly media and basin refreshments. At the end of the experiment, all slides were examined using fluorescent microscopy. Counts were made and the longest dimension of the five largest *S. latissima* developmental stages recorded. From these, the survival rate (%) of the settled meiospores, germination rate (%) and mean size was calculated. These slides were then exposed to the flume under the conditions described above and re-analysed using fluorescent microscopy.

Experiment three (E3)

100,000 meiospores were distributed into borosilicate basins containing 150 mL of F/2 + 0.125 mL $GeO_2 \cdot L^{-1}$ and a glass slide with Kuralon twine (ϕ 2.5 mm) wound ten times around and secured using cyanoacrylate glue. These were then cleaned using the procedure for the slides. Seven combinations were examined: seawater with and without: F/2, GeO_2, or slide pre-treatment with (20.7±2.4 $mg \cdot cm^{-2}$) yeast extract (Marmite©, Unilever plc, UK; n=5; Fig 1/2).

These were settled for 48 hours in the dark at 8.5 °C, then transferred into fresh borosilicate basins containing the respective media with gentle bubbling. These were cultured for six weeks with weekly media and basin refreshment. For the first week, illumination was by blue fluorescent lighting at 15-25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ 12:12 L:D. This was then increased to 30-50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ to encourage growth.

After four weeks, fluorescent microscopy was used to make counts, measure size and estimate % cover. Slides were photographed through a stereomicroscope and measurements were made of the ten largest sporophytes using ImageJ 1.45s (National Institutes of Health, USA). Incubations then continued for a further two weeks, in which all treatments were refreshed with F/2 media (Fig 1c). After six weeks the experiment was ended and measurements were repeated. In addition, cover was estimated and then all sporophytes were scraped from the twine, dried and weighed.

Minitab v15 (Minitab Inc) and Excel 2010 (Microsoft) were used for all statistical analyses. ANOVA (AN), nested ANOVA (nAN) and 2 way ANOVA (2wAN) were used where the Anderson-Darling test for normality (Anderson and Darling 1952) was satisfied. Where nAN or AN were not appropriate, pseudo-replicated data was averaged and a Mann-Whitney U tests (MW) was used.

Human and Animal Rights

No humans or animals were used or harming in the following experimentation.

RESULTS

Experiment 1 (E1)

Settlement during 48 hours in darkness was significantly greater by 170 % than when illuminated under a 12:12 hour light cycle (AN: $p<0.0001$; Fig 3a).

Experiment 2 (E2)

Settlement in non-enriched seawater was 40 % lower (AN: $p<0.05$) than in F/2 (Fig 3b). Settlement on the pre-treated surface was increased by 16 % (AN: $p<0.05$). Neither the presence of GeO_2 or a roughened surface affected settlement ($p>0.05$).

229 Exposure to the flume significantly reduced the number of meiospores in all conditions by 44 - 70 %
230 (2WAN: $p<0.0001$). Following flume exposure, only the meiospore density in non-enriched seawater
231 was significantly different to the standard, being 50 % lower (AN: $p<0.01$).
232
233 After three weeks of culturing, the survival of the settled meiospores was 71 % lower in the absence
234 of nutrient media (AN: $p<0.0001$; Table 1). Survival was boosted 66, 38 and 72 %, respectively by the
235 pre-treatment (AN: $p<0.005$), 200 and 400 µm particle roughened slides (AN: $p<0.05$ and MW:
236 $p<0.05$, respectively). Germination success was significantly reduced in non-enriched seawater (MW:
237 $p<0.001$) and in the GeO_2 treatment (nAN: $p<0.05$) by 15 and 5 %, respectively.
238
239 Mean size was not affected by the surface roughness ($p>0.05$) but was significantly reduced in non-
240 enriched seawater (Table 1; MW: $p<0.005$) and by the presence of GeO_2 (nAN: $p<0.0001$) by 63 and
241 40 %, respectively. This was seen as a shift in the size distribution (Fig 3). Yeast extract pre-treatment
242 increased mean size by 28 % (AN: $p<0.0001$).
243
244 Flume exposure after three weeks, did not lead to any significant change in the number or size of *S.*
245 *latissima* ($p>0.05$).
246
247 **Experiment 3 (E3)**
248 **Effect of yeast extract surface pre-treatment**
249 After four weeks of cultivation in non-enriched seawater, the pre-treatment led to a 70 % reduction
250 in the counts of *S. latissima* (BvC; MW: $p<0.05$). A non-significant reduction was seen with
251 pretreatment in F/2 media (CvF; $p<0.05$). The % cover was not significantly affected ($p>0.05$),
252 neither was the size of the sporophytes by the absence of nutrient media. In F/2, yeast extract led
253 to sporophytes which were 17 % smaller than the control (EvF; nAN: $p<0.0001$). After six weeks,
254 basins which initially had no nutrient media had 49 % lower counts of *S. latissima* then when pre-

255 treated (BvC; nAN: $p<0.05$), while their size was not affected ($p<0.05$). However, when always grown
256 in F/2, the sporophytes were 37 % larger with the pre-treatment (EvF nAN: $p<0.001$), while % cover
257 and final dry weight were not affected ($p>0.05$).
258
259 Overall, the yeast extract led to reductions in the counts of *S. latissima* and slowed development in
260 the first four weeks. After six weeks, counts were still lower, but larger sporophytes with less
261 consistent coverage had been able to develop, leading to a similar end point biomass.
262
263 **Effect of Germanium dioxide**
264 Counts and % cover after four weeks were not affected by the presence of GeO_2 ($p>0.05$). The size of
265 *S. latissima* grown without nutrient media was also not affected. Those cultured in F/2 with 9 day
266 GeO_2 exposure were 17 % larger (EvG; nAN: $p<0.0001$) than those without GeO_2.
267
268 After six weeks, slides initially grown without nutrient media (A-C) still had no difference in their
269 counts ($p>0.05$), but were 52 % smaller without a 2 day GeO_2 exposure (AvB; nAN: $p<0.0001$). Those
270 always grown in F/2 showed no difference in the final dry weight or their size due to GeO_2 ($p>0.05$)
271 but had a slightly higher % cover after 9 days exposure rather than 2 days (EvG; AN: $p<0.05$).
272
273 Overall, a short period of GeO_2 lead to increased growth rate and a more even distribution, even
274 though the final biomass achieved was not affected.
275
276 **Effect of Nutrient media**
277 Nutrient media had the most impact on the growth of *S. latissima*. After both four and six weeks,
278 macroscopic growth was only evident in conditions containing F/2 and were 2 orders of magnitude
279 larger than without it (Figure 5; MW: $p<0.00001$). In addition after 4 weeks, counts were
280 unequivocally higher in nutrient media incubations; 69 % with a 2 day GeO_2 exposure (BvE; nAN:

281 $p<0.005$), 48 % without GeO_2 (AvD; nAN: $p<0.001$) and 185 % with the pre-treatment (CvF; nAN:
282 $p<0.0001$).
283
284 Nutrient media introduction to A, B and C after four weeks led to a large increase in the counts by
285 week six (Figure 5; Table 2); These were 217 and 205 % in the pre-treatment (C) and GeO_2 condition
286 (B), but only 87 % in the non-GeO_2 condition (A). The size change followed a similar pattern with
287 increases of 522, 308 and 78 % for C, B and A respectively.
288
289

DISCUSSION

LIGHT

In E1, settlement of *S. latissima* meiospores was 170 % higher over 48 hours of darkness rather than under a 12:12 light cycle, and so darkness is recommended to maximise settlement. Dark settlement has been used before in other *S. latissima* experimentation, although no explanation was given (Shea and Chopin 2007). It may be that settlement is stimulated by darkness, as has been observed in *Ulva clathrata* (P Kerrison unpub. results). Then again, it may also be that extended periods of darkness force settlement; Swimming behaviour increases the dispersal capacity of meiospores and is fuelled by both lipid reserves and possibly also photosynthesis (Reed et al. 1999). As in other kelps, *S. latissima* meiospores contain a chloroplast and so may use photosynthesis to maintain swimming in the light. It has been observed that *Pterygophora californica* lipid reserves become depleted during dark periods (Reed et al. 1999), and that in both *P. californica* and *Macrocystis pyrifera,* 48 hours of darkness sharply decreases the rate of swimming (Reed et al. 1992).

Light exposure can also reduce settlement competency through photodamage. High irradiance levels (>300 $\mu mol \cdot m^{-2} \cdot s^{-1}$) are known to reduce the settlement and/or germination success *of P. californica* and *M. pyrifera* (Cie and Edwards 2008; Graham 1996). Even exposure to only 75 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 1-12 hours has been shown to decrease settlement in *P. californica* and germination success in *M. pyrifera* (Cie and Edwards 2008). These facts, combined with the well described germination of kelp meiospores in darkness (Huovinen et al. 2000; Han et al. 2011; Reed et al. 1992), helps to explain why continuous darkness leads to more successful settlement of *S. latissima* meiospores.

NUTRIENTS

Nutrient enrichment consistently increased settlement in both E2 and E3, similar to findings in *Pterygophora californica* (Amsler and Neushul 1990). This is thought to be an adaptation to facilitate

316 settlement in suitable benthic microhabitats (Amsler and Neushul 1989). It may also be that a
317 certain meiospore nutrient quotient is required before successful settlement is achieved, so higher
318 nutrient concentrations lead to greater settlement. Spore release and settlement in nutrient media
319 has been recommended for the hatchery cultivation of kelp by Flavin *et al*. (2013).
320
321 But, very high concentrations can be inhibitory, deterring settlement and causing negative
322 chemotaxis in kelp meiospores (Amsler and Neushul 1989). Conversely, insufficient nutrients can
323 slow or stall the development of microscopic sporophytes and gametophytes (Hoffman and
324 Santelices 1982; Kinlan et al. 2003; Reed et al. 1991). This appears to be the case in E2, where
325 nutrient enrichment lead to increased survivorship, increased germination and more growth
326 observed after three weeks. Similarly, in the fourth week of the E3, increased sporophyte density
327 and faster development was seen in the presence of nutrient enrichment. In fact, the benefit of F/2
328 media on development was so great that all enriched samples had sporophytes visible to the naked
329 eye (ca 1 mm), while all non-enriched samples were still microscopic (0.02 mm). This agrees with
330 previous investigations where the fertilised hatchery growth of *Undaria pinnatifida* for ca 6 weeks,
331 lead to faster development of adult sporophytes following outplanting at a cultivation site (Gao et al.
332 2013). However, it should be noted that there was a confounding effect of temperature control in
333 that study.
334
335 In E3, the introduction of F/2 to the nutrient depleted conditions A, B and C for the final two weeks,
336 greatly enhanced growth, so that the number and size of sporophytes increased 2-3 and 2-6 times
337 respectively. After 6 weeks, these had reached a similar developmental stage as the continuous F/2
338 treatments (D-G) after only 4 weeks. This shows the importance of constant or continuing nutrient
339 enrichment within the kelp hatchery to enhance the growth of the developing sporophyte, and so
340 minimise development time. A similar enhancement has been shown by nutrient enrichment
341 through *M. edulis* hatchery co-culture (Rößner et al. 2014).

342

343 It is interesting to see that the counts·mm^{-2} in A, B and C at 4 weeks, increased following the nutrient
344 enrichment. Given the complex 3D surface of twine, small structures such as meiospores and
345 unbranched gametophytes are easily missed, even using fluorescent microscopy, and so the counts
346 at week 4 are likely to be underestimated. Therefore the increased counts at week 6, maybe partly
347 explained by the growth of missed meiospores into larger more obvious visible structures. However,
348 the counts for A and B were even larger than was seen in D-G at a similar developmental stage
349 (week 4). It is therefore also possible that growth with low nutrients, stimulated increased branching
350 in the developing gametophytes, resulting in larger multi-branched females which gave rise to
351 increased numbers of embryonic sporophytes. This suggests that counterintuitively, a low nutrient
352 period, increases the final sporophyte density, although the cost is an increased development time.
353 The re-supply of nutrients may also lead to more rapid sporophyte development as has been
354 documented in four kelp species (Carney 2011), as the juveniles maybe 'primed' to take advantage
355 of any increase in nutrients.

356

357 **GERMANIUM DIOXIDE**

358 Shea and Chopin (2006) show that a dose of 0.1 - 0.5 mL of saturated $GeO_2 \cdot L^{-1}$, applied after 8 days,
359 eliminated almost all diatoms and allowed optimal *S. latissima* sporophyte development over a 40
360 day experiment. We tested a dose within this range (0.25 mL·L^{-1}) and found that its continuous use
361 over 3 weeks lead to a 40 % decline in growth. This agrees with other reports on the inhibition of
362 growth and slowed development seen in the presence of GeO_2 (Markham and Hagmeier 1982;
363 Mizuta and Yasui 2012). Therefore, we wanted to test whether exposure for only a few days at the
364 start of the cultivation period was more favourable, similar to the method of Lüning (1982), who
365 applied a dose of 2 mL·L^{-1} over the first four days.

366

In E2, it was found that settlement was not influenced by GeO_2 and so it can be safely used during this period with no ill effects. Further to this, in E3, it was found that a 9 day treatment of GeO_2 resulted in larger sporophytes than only 2 days. No significant differences in size were observed after six weeks, however, cover was higher after the 9 day treatment. It therefore appears that exposure for 9 days leads to the most favourable results, and should eliminate the threat posed by overgrowth by benthic diatoms during the early development of *S. latissima* meiospores.

YEAST EXTRACT

Conditioning of the settlement surface with yeast extract was hypothesised to be beneficial to the settlement of algal spores due to its complex composition and the presence of potential settlement cues (Santelices and Aedo 1999; Lee et al. 2008), while its current commercial value as a condiment would make it easily and cheaply available for hatchery use. As expected, it significantly increased spore settlement in E2, and in addition at the end of three weeks, both survivorship and size were significantly enhanced relative to the control. This was unexpected as the amount of yeast extract applied to the slide was very low and appeared to dissolve quickly into the media, which would be lost when the media was exchanged at the end of the settlement period. However, it appears that some beneficial component/s was either absorbed by the meiospores during the settlement period, or remained within the slide boundary layer despite the transfer into fresh media. While the identity of this component/s is currently unknown, possible candidates are vitamins such as B12 and trace metals such as iron. The presence of these components on the slide surface may have also boosted settlement (Amsler and Neushul 1990).

Another possibility is that juvenile *S. latissima* are partially heterotrophic and are making use of the abundant amino acids and complex carbohydrates present in the extract (www.Foodcomp.dk); or that bacterial populations that heterotrophically consumed the yeast extract and releasing a

392 component beneficial to *S. latissima*. Despite the cause being unknown, the results from E2 show
393 that this pre-treatment is beneficial and cost-effective, however E3 did not agree.
394
395 In E3, a thicker layer (20.7±2.4 mg·cm^{-2}) was applied to the twine than was used on the slide
396 (1.6.7±0.5 mg·cm^{-2}) to ensure an even coverage. However, this 13 fold increase in dosage led to a
397 negative effect on the development of *S. latissima* meiospores, with a reduction in sporophyte
398 counts and size after 4 weeks. Much of the applied extract is assumed to have soaked into the
399 structure of the twine and so would have taken far longer to dissolve into the media than when
400 applied to the glass slides in E2. This could have resulted in inhibitory concentration (Amsler and
401 Neushul 1989) of certain nutrients at the surface of the twine, so reducing settlement and survival.
402 In addition, the high organic matter content, may have led to a bloom of heterotrophic bacteria on
403 the twine surface smothered the developing meiospores and gametophytes. Such bacterial
404 overgrowth has been observed if the media is not exchanged, two days following the introduction of
405 spore solution (P Kerrison unpub results). This is thought to be due to labile organics released from
406 the adult sporangia along with the meiospores.
407
408 After 6 weeks the E3 yeast pre-treatment, lead to a more sparse and patchy sporophyte population.
409 While dry weight biomass was found to be not different between conditions, the sporophytes were
410 largest where the pre-treatment had been applied (F). This is not thought to be due to the pre-
411 treatment *per se*, but due to the reduction in density its over-application caused. By reducing
412 competition, it allowed the remaining sporophytes to grow faster achieving a larger size after 6
413 weeks (Reed et al. 1991; Steen and Scrosati 2004). Such a patchy distribution needs to be avoided
414 for best success of the outplanted twines, however, and the faster growth between week 4 and 6 on
415 the lowered density twines that received the pre-treatment reveals that the settlement density used
416 in these study are likely to be too high. To minimise the hatchery time, the optimal density of
417 meiospores should be settled. This will be a trade-off between limiting the interspecific competition

between compatriots, slowing overall grow, and ensuring good coverage of sporophytes on the lines. It is also likely that a high density of recruits will be more resilient and likely to success compared to a low density, where disturbance is more likely to lead to partial crop failure. The effect of settlement density on development time and outplanting success warrants future experimentation.

ROUGHNESS

Surface roughness and topography is known to be an important factors affecting the settlement of many benthic organisms, including macroalgae (Kohler et al. 1999; Bers and Wynne 2004). The roughening of the glass slide had no effect on the settlement of *S. latissima* and no preference for settlement within particular features was observed (data not shown). In the green alga *Ulva* spp., zoospore settle preferentially on particular topographic features; aggregrating in crevices, depressions and at the intersection of dissimilar features (Schumacher et al. 2007; Long et al. 2010). The lack of an effect of roughness on settlement density or distribution here (data not shown) indicates that *S. latissima* meiospores do not show topographic selectivity at the roughness scale used in this study. Further to this, variation in roughness, did not affect the removal rate of meiospores when exposed to high flow within the flume. This is probably because boundary layer characteristics were not substantially different between the different roughnesses examined.

After three weeks of cultivation, survivorship was substantially boosted on the roughest surfaces by 38-72%. This may simply be because the rougher slides had a higher surface area, allowing more sporophytes to develop with less competition for space. Additionally, surface roughness may have affected holdfast development, as is seen in the red macroalgae *Polysiphonia* sp (Woods and Fletcher unpublished). Roughened areas are thought to improve the attachment strength in developing juvenile, due to the increased attachment area for the holdfast and the physical locking of holdfast rhizoids into microscopic crevices (Milligan and DeWreede 2000; Morrison et al. 2009).

Therefore, the improved survival seen maybe a consequence of less accidental detachment due to generally stronger attachment in the developing juveniles.

CONCLUSIONS

This study provides information on how the settlement of *S. latissima* meiospores and their development into juvenile sporophytes is affected by light, nutrients, GeO_2, surface conditioning with a yeast extract and surface roughness. Through these simple manipulations of the hatchery conditions, faster growth, increased survival and consistent coverage is achievable on seeded twine of *S. latissima*. This study has shown that:

1. meiospore settlement should be conducted in darkness to maximise settlement.
2. nutrient enrichment should be used throughout the hatchery phase as this will improve settlement, survival, germination and maximise growth.
3. GeO_2 should be used for the first 9 days as this will inhibit early competition from diatoms, so leading to greater growth and highly consistent coverage.
4. A rougher surface also improves survival, which is thought to be due to improved attachment of developing sporophytes.
5. The use of yeast extract surface pre-treatment shows promise and can improve both survival and growth. However, it is not recommended until further research has been conducted to determine the correct dosage.

While this study examined only *S. latissima*, it is highly likely that these results will be applicable to the hatchery cultivation of other kelp species such as *Laminaria digitata*.

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473
474 **CONFLICT OF INTEREST**
475 None declared.
476
477
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611

612 **Figure 1** Summary of experiments 1-3 (E1-3). In E1, settlement was examined in either the light or
613 dark. In E2, dark settlement was examined in either nutrient media (F/2) or seawater (NW), with or
614 without germanium dioxide (GeO_2) or yeast extract pre-treatment (PT) and with three roughness's:
615 light, medium or coarse (L, M or C rgh). This was followed by either continued cultivation, or
616 examination with or without flume exposure. In E3, settlement was examined in larger vessels, with
617 several conditions of nutrients, GeO_2 and yeast extract. In all, cultivation was continued for a further
618 6 weeks, with all cultures grown in F/2 for the last 2 weeks (n=5)

619

Figure 2 Conditions examined in experiment 3 (A-G). These are combinations of: seawater or F/2 enriched seawater (NW or F/2), pre-treatment with a yeast extract (PT) and application of germanium dioxide for either 2 or 9 days (GeO_2 2d or 9d). Horizontal black arrows compare enriched and non-enriched seawater, light grey vertical lines compare the use of pre-treatment while dark grey lines compare GeO_2 application and duration

Figure 3 Densities of *Saccharina latissima* meiospores after settling for 48 hours onto glass slides under different conditions a) Experiment 1: F/2 media under either: 20-30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ 12:12 L:D (Light) with 0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Dark). b) Experiment 2: Comparing dark settlement in F/2 media (F/2), seawater (NW), in F/2 including 0.56 $mg \cdot L^{-1}$ germanium dioxide (GeO_2), in F/2 on slides pre-treated with a yeast extract (PT) and in F/2 on slides roughened with three grades of sandpaper: particle size 100-400 µm (L, M and C rgh respectively). Statistical significance: *p<0.05, ****p<0.0001

Figure 4 Frequency distribution of *Saccharina latissima* size in experiment 2. Three weeks following settlement onto glass slides under different conditions. Comparing F/2 media (F/2), non-enriched seawater (NW), including 0.56 $mg \cdot L^{-1}$ germanium dioxide (GeO_2), on slides pre-treated with a yeast extract (PT) and on slides roughened with 200 µm particle size sandpaper (Mrgh). Data bins all pseudo-replicates

Figure 5 Variation in size (µm) and density (mm^{-2}) of *Saccharina latissima* between incubation week four and six of experiment 3 in each experimental condition (A-G). The week six density for E-G could not be determined and so their horizontal position may not be accurate

Figure 6 Frequency distribution of *Saccharina latissima* size in experiment 3. Six weeks following settlement onto Kuralon twine under different conditions. Comparing culturing in F/2 media (F/2), 2

645 or 9 day exposure to 0.56 mg·L^{-1} germanium dioxide (GeO_2 2/9d) and pre-treatment with a yeast
646 extract (PT). Data bins all pseudo-replicates
647
648 **Table 1** The characteristics of *Saccharina latissima* three weeks following settlement, in experiment
649 2. All treatments are compared to the control, which contains only F/2. Statistical significance:
650 *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, Mann-Whitney U Test (MW), ANOVA (AN), nested
651 ANOVA (nAN)
652
653 **Table 2** Overview of experiment 3 results on growth of *Saccharina latissima* in conditions A-G. NW-
654 Natural seawater, GeO_2– 0.56 mg·L^{-1} germanium dioxide exposure for 2 or 9 days (2/9d), F/2 –
655 nutrient enrichment with F/2, PT – pre-treatment with yeast extract
656

Fig 1
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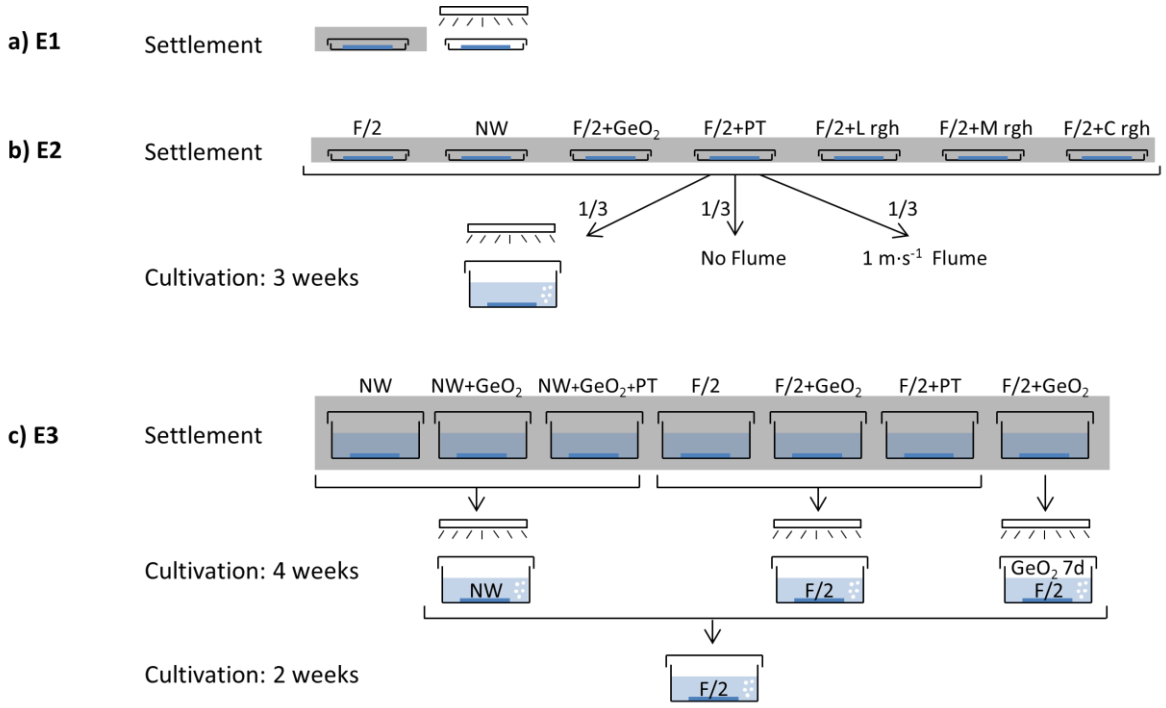

Fig 2
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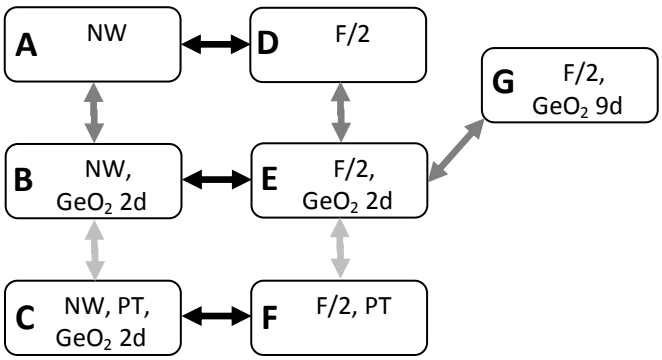

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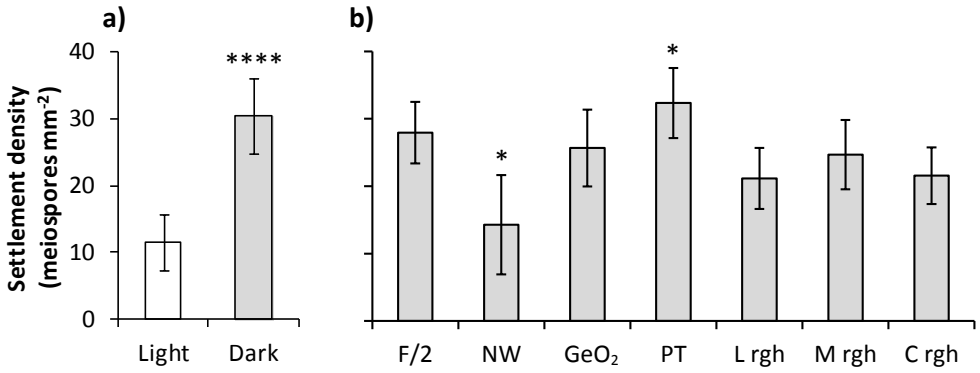

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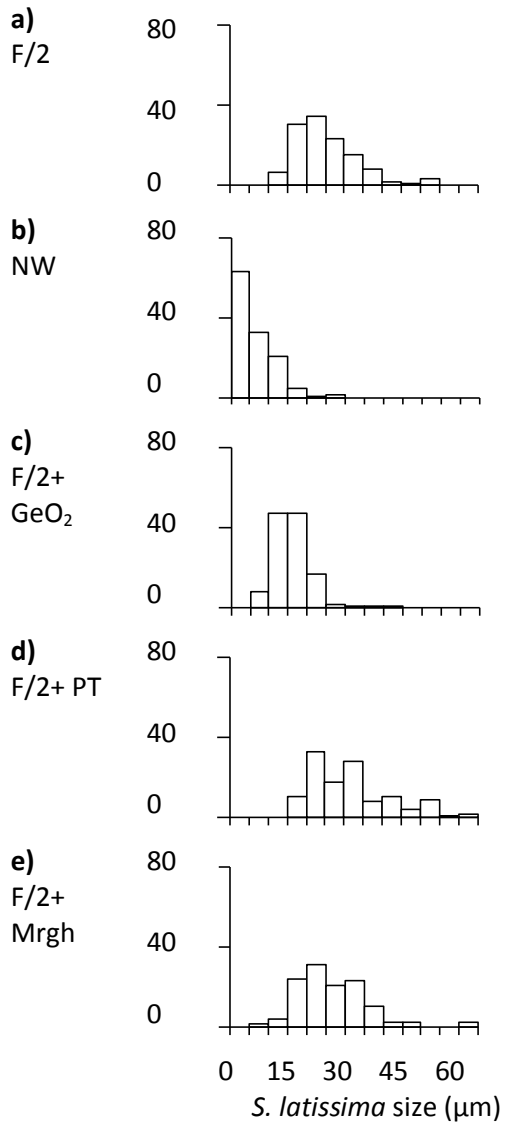

Fig 5

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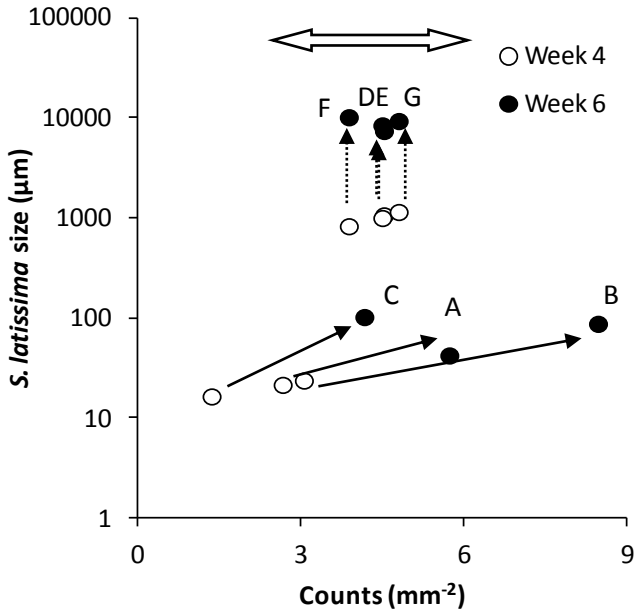

Fig 6
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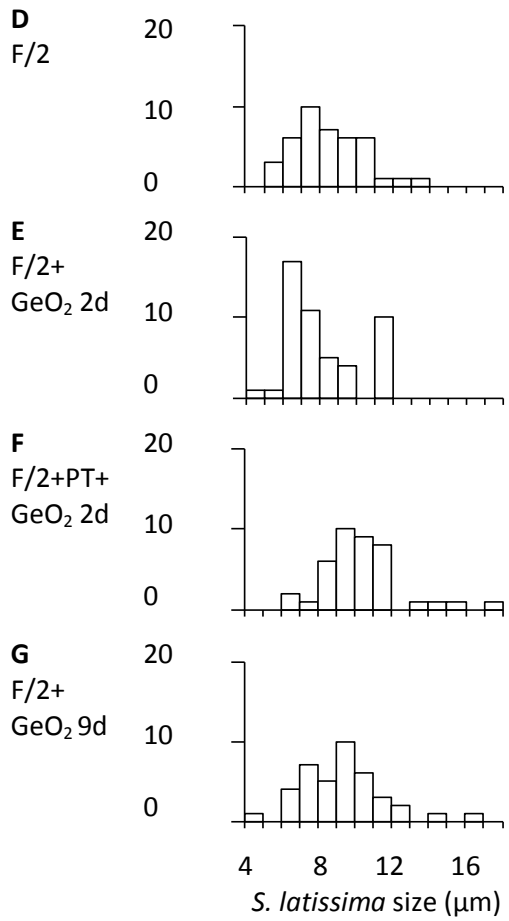

Figure 6 Frequency distribution of *Saccharina latissima* size in experiment 3. Six weeks following settlement onto Kuralon twine under different conditions. Comparing culturing in F/2 media (F/2), 2 or 9 day exposure to 0.56 mg·L^{-1} germanium dioxide (GeO_2 2/9d) and pre-treatment with a yeast extract (PT). Data bins all pseudo-replicates

table 1
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	Survival (%)	Δ vs. control (%)	**Germinated (%)**	Δ vs. control (%)	**Size (µm)**	Δ vs. control (%)
F/2 (control)	16 ±4	----	92 ± 4	----	23 ± 3	----
NW	5 ± 3	- 71 ^{****AN}	79 ± 9	- 15 ^{*MW}	9 ± 1	- 63 ^{**MW}
GeO_2	14 ± 12	ns	88 ± 4	- 5 ^{***nAN}	14 ± 2	- 40 ^{****AN}
PT	26 ± 7	+ 66 ^{***AN}	91 ± 2		29 ± 5	+28 ^{**AN}
L rgh	15 ± 8	ns	86 ± 7		25 ± 2	
M rgh	21 ± 4	+ 38 ^{*AN}	88 ± 7		23 ± 3	
C rgh	27 ± 9	+ 72 ^{*MW}	92 ± 3		25 ± 6	

table 2
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Condition		4 weeks			6 weeks			
		Counts (mm^{-2})	FOV cover (%)	Size (µm)	Counts (mm^{-2})	Twine cover (%)	Size (µm)	Dry weight (mg)
A	NW	3.1 ± 1.9	n/a	24 ± 13	5.8 ± 5.4	n/a	43 ± 14	0
B	NW, GeO_2 2d	2.7 ± 0.8	n/a	22 ± 7	8.5 ± 4.2	n/a	89 ± 17	0
C	NW, PT GeO_2 2d	1.4 ± 0.1	n/a	17 ± 5	4.2 ± 1.4	n/a	103 ± 25	0
D	F/2	4.5 ± 0.7	72 ± 13	1080 ± 220	n/a	99.8 ± 0.5	8520 ± 1500	33 ± 8
E	F/2, GeO_2 2d	4.5 ± 0.5	70 ± 6	1010 ± 110	n/a	98.2 ± 1.3	7500 ± 630	42 ± 18
F	F/2, PT, GeO_2 2d	3.9 ± 0.2	38 ± 24	840 ± 280	n/a	96.6 ± 3.9	10280 ± 720	36 ± 0
G	F/2, GeO_2 9d	4.8 ± 0.8	75 ± 13	1160 ± 270	n/a	99.3 ± 1.0	9410 ± 1600	36 ± 13